Final report for the "Brine Shrimp Kinetics Study, Project 5"

Summary

Introduction

Selenium has long been recognized as a reproductive toxicant[16,34] causing teratogenesis and chick mortality in birds[2]. The primary piscine and avian exposure pathway for selenium is the diet[1,23,24,28,33]. Consequently, environmental regulations protecting animals from selenium exposure ought to aim at maintaining selenium concentrations in relevant prey organisms below effect threshold concentrations. As such, a tissue residue criterion (TRC) rather than traditional water-quality criteria has recently been proposed for selenium by the USEPA[42].

A TRC approach, however, is sensitive to variation in bioaccumulation of the element in question, which potentially varies with site-specific conditions (water and sediment chemistry) and other environmental factors. Furthermore, bioaccumulation of chemicals is often species specific and may be subject to homeostatic control, complicated uptake kinetics and excretion or elimination in the organisms of interest[1,6,9,30,40]. Similar concerns apply to standard water quality criteria and a TRC approach combined with biodynamic modeling provide an elegant solution to these problems because steady state concentrations can be predicted from measured rates of uptake and elimination in the organism of interest[8,30] (see below for details).

Great Salt Lake, Utah is an important staging, wintering, and breeding area for many migratory waterfowl and shorebirds. The high salinity of Great Salt Lake (3-10 times that of seawater) limits the aquatic fauna; the highly abundant brine shrimp, *Artemia franciscana*, is the largest aquatic predator/consumer and serves as one of the principal avian food sources[12,26]. Because of the unusual water chemistry in Great Salt Lake, standard water quality criteria do not apply and a TRC approach is currently applied for selenium discharge from the Kennecott copper smelting facility. Using an estimated dietary effect threshold for avifauna of 5 mg/kg dw[23,41] and *in situ* measurements of total waterborne selenium concentrations and corresponding concentrations in brine shrimp[5], the current water quality discharge limit is set at 27 µg/L. The *in situ* measurements from the Kennecott copper smelting facility[5] provide a site-relevant foundation for the establishment of discharge limits in providing a relationship between ambient selenium concentrations and selenium accumulation in *Artemia* but are associated with some uncertainty. This uncertainty is a consequence of relatively limited field-derived data with low resolution of exposure concentrations (consisting of waterborne selenium concentrations below 5, around 30, and >80 µg/l, L) at concentrations that appear to bracket the "knee" in the selenium accumulation curve[5] (see Fig 7 for definition of the term "knee"). The data forming the basis for the current discharge limit were analyzed by simple linear regression (which errs on the conservative side) yielding predicted brine shrimp selenium concentrations of 5 mg/kg dw at 27 µg/l. Despite the conservative approach taken in the analysis these data, it is important to recognize that considerable uncertainties remain. The data set is small, exposure times are uncertain and life stages of

the brine shrimp varied. Considerable error may therefore be associated with this estimated “safe” level.

The main objective of the present study was to provide reliable predictions of selenium accumulation in *Artemia franciscana* under conditions realistic for the populations residing in the Great Salt Lake (GSL), Utah. Controlled laboratory experiments were performed to address this uncertainty and to better define the relationship between ambient selenium concentrations and concentrations of accumulated selenium in brine shrimp.

This main objective was addressed by pursuing the following specific objectives:

1) Determine the influence of salinity on selenium uptake and feeding rate by *Artemia franciscana*.
2) Determine selenium uptake rates in *Artemia franciscana* from dissolved selenium concentrations in artificial GSL water (uptake kinetics).
3) Determine dietary selenium intake and subsequent selenium assimilation efficiency in *Artemia franciscana* fed a diet of selenium-loaded algae cells (*Dunaliella viridis*).
4) Determine selenium elimination rates from *Artemia franciscana* following selenium accumulation from elevated ambient concentrations.

5) Model selenium accumulation in *Artemia franciscana* based on the results from objectives 1-3 to provide predictions of selenium accumulation during realistic exposure scenarios.
6) Determine the "knee" of the dissolved selenium accumulation rate curve in *Artemia franciscana*.
7) Investigate possible regulation of selenium accumulation in *Artemia franciscana* during prolonged exposure to selenium.

Materials and Methods

Organisms

The algae *Dunaliella viridis*, which is indigenous to the GSL and available for mono-culturing, was used in the present project and was obtained as a gift from Marjorie Brooks (then at the University of Wyoming). Cultures of *D. viridis* were maintained in artificial GSL medium (Table 1) for the present project and subcultures were raised in appropriate selenium concentrations as described below. The brine shrimp, *Artemia franciscana*, were obtained as cysts from M&M Suppliers, Bothell, WA and were hatched in natural seawater from Bear Cut Florida. Salinities in the Great Salt Lake are reported to range from 125 to 142 (parts per thousand [ppt]). However for practical reasons, *Artemia* were maintained in bulk culture at 73.5 ppt salinity artificial GSL water. For subsequent experiments examining Se uptake at higher salinities (100 and 160 ppt), *Artemia* were acclimated to these salinities in artificial GSL waters for a period of at least 48 hours prior to experimentation. *Artemia* were maintained in mass culture in 4 individual aerated 10-gallon tanks with partial water renewal as necessary and were fed

commercially available dried algae daily. Specifically, 1 gram of Wardley Premium Algae Discs (Secaucus, NJ) was homogenized in 20 mls of deionized water and offered to artemia according to culture density. Feeding amount was adjusted such that artemia were able to completely clear the water between feedings.

General experimental procedures

Only adult (>4 mg whole animal wet weight) artemia were used in the present experiments and a radioisotope labeling procedure was employed to facilitate fast, yet accurate measurements of low levels of selenium in a highly saline matrix. ^{75}Selenium as selenate (specific activity) was obtained from the University of Missouri, Columbia research reactor. Secondary ^{75}Se stock solutions (of varying specific activity serving experimental needs) were prepared in deionized water for individual experiments, and the specific activity of these stock solutions were verified by measuring the total selenium concentrations by graphite furnace atomic absorption spectrometry (see below) and the 75Se radioactivity. From the ratio of ^{75}Se radioactivity and total selenium concentration in the stock solutions (specific activity; SA) and corresponding ^{75}Se radioactivity from artificial GSL water, algae and artemia, selenium concentrations were determined as:

^{75}Se radioactivity (cpm) / SA

And

^{75}Se radioactivity (dpm) * (cpm/µg Se) = µg Se

	Artificial GSL for culturing (g/L)	Artificial GSL for experiments (g/L)	Algae Media (g/L)
NaCl	50.960	69.306	52.596
$MgCl_2$ ($6H_20$)	10.572	14.378	1.500
$MgSO_4$ ($7H_20$)	8.628	11.734	0.500
KCl	2.632	3.580	0.200
$CaCl_2$ ($2H_20$)	0.147	0.200	0.200
$NaHCO_3$	0.164	0.223	0.043
$CaSO_4$ ($2H_20$)	0.397	0.540	--
KNO_3	--	--	1.000
KH_2PO_4	--	--	0.035
Trace Metals	--	--	10 ml/L
Iron Solution	--	--	10 ml/L

Table 1. *Composition of artificial GSL media and D. viridis culture medium*

Analytical procedures

Gamma activity arising from the Se-75 isotope was detected directly on undigested, sometimes alive material, in water or in dried samples by a Packard, Cobra II auto gamma counter D5003 using a counting window from 60-467 keV. Total selenium concentrations in stock solutions were determined by graphite furnace atomic absorption spectrometry (Varian, 220Z). A sample injection volume of 10 µl and 10 µl of modifier (1 mg Ni/ml) were co-injected with deionized water for a total injection volume of 25 µl. Following evaporation, an ashing step of 1000°C for 8 seconds preceded the atomization step of 2600 °C (detection limit: 0.6 µg/L). Absorbance recorded from samples was compared to the absorbance obtained from automatically generated dilutions of a certified selenium standard (Aldrich) to determine selenium concentrations.

Algal dry weight was determined by filtering 15 mls of an algae culture of known cell density (determined by cell counting on a hemacytometer) through a pre-weighed 47-mm

glass microfibre filter (Whatman) and then rinsing the filtered cells with 10 mls of 0.5 M ammonium formate to remove high density salts. The filters were then dried for 24 hrs at 80 °C and re-weighed. Dry weight was estimated by dividing mass of the dried algae by the number of algae cells in the 15 ml sample.

Artemia dry weight was determined by sampling and gently blotting dry 12 adult artemia on a paper towel, then placing them on 1-cm^2 pieces of pre-weighed aluminum foil. These artemia were weighed to determine wet weights, then dried for 24 hours at 80 °C and re-weighed to determine dry weights.

Data presentation and statistical evaluation

Data are reported as means ± SEM throughout. Non-linear regressions were performed using SigmaPlot version 8.0 and statistical comparisons were performed using Student's two-tailed *t*-tests.

Influence of salinity on selenium uptake from the water and feeding rates (objective 1)

To examine the effect of salinity on selenium uptake from the water, 24-hour and 48-hour selenium uptake was measured in individual artemia at 100 and 160 g/L GSL medium at 1.75 ± 0.05 and 1.83 ± 0.08

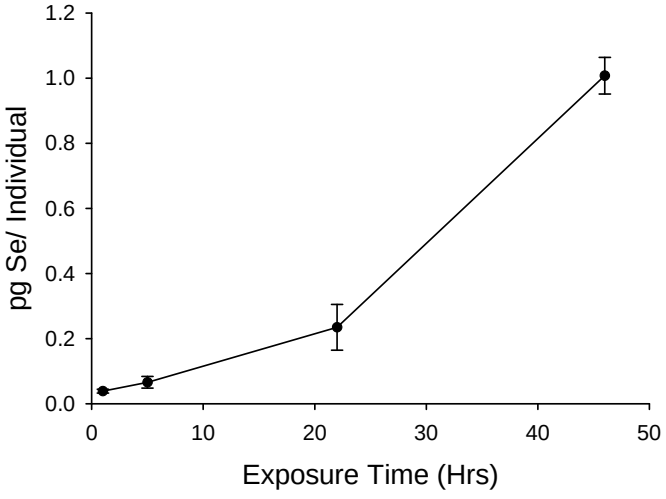

Fig. 1. Selenium accumulation in artemia during a 48-hour exposure to 13 ng Se/L as ^{75}Se-labeled selenate. Values are expressed as pg Se/individual. Mean ± SEM, n=10.

µg Se/L respectively. Measurements were performed in triplicate treatments, each with 15 adult artemia and otherwise as outlined in the SOP provided below. A 24-hour exposure duration was chosen based on experiments demonstrating linear Se accumulation for at least 24 hours (Fig.1) and initial experiments demonstrated that Se exposure concentrations remained relatively constant over at least 24 hours of exposure (Fig. 2).

Feeding rates were also determined in adult artemia fed *D. viridis* at 100 and 160 g/L GSL medium. Adult artemia (n= 15) in 30 mls of GSL media were offered a density of 93.4 $\cdot 10^6$ *D. viridis* cells and cell density was monitored every 10 minutes for a total of 30 minutes by measuring the absorbance of water samples at 750 nm. Preliminary experiments revealed a good correlation between cell count (cells/mm^2) and absorbance (Fig. 3). The feeding rate experiments were performed in triplicate and otherwise as described in the SOP.

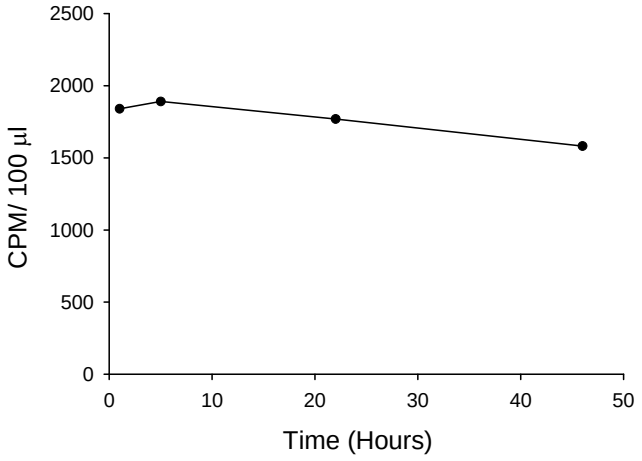

Fig. 2. Levels of ^{75}Se in GSL medium during a 48-hour exposure of artemia to 13 ng Se/L as selenate under conditions employed in the present study (see text for details).

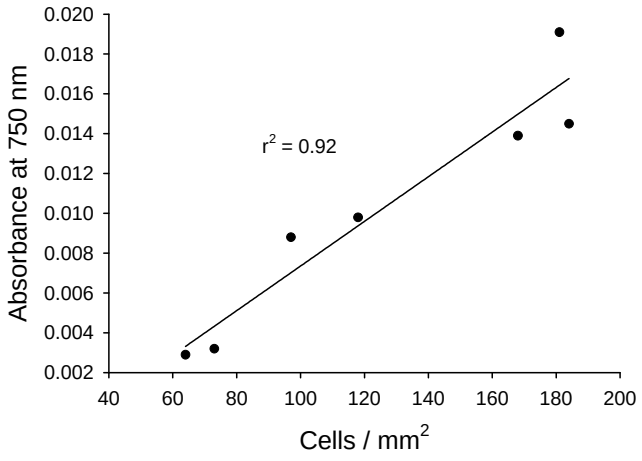

Fig. 3. Correlation between absorbance at 750 nm and cell count. See text for further details.

Determination of selenium uptake rates in brine shrimp from dissolved selenium concentrations in GSL water (Objective 2 & 6)

Based on findings from the salinity experiments above, all subsequent experiments were performed at 100 g/L unless otherwise stated. Adult artemia (n=15) were placed in 25 ml

artificial GSL medium in 50-ml PYREX glass beakers that were gently aerated to ensure oxygenation and mixing and were exposed to ^{75}Se (as selenate) for 24 hours at nominal concentrations ranging from 1 to 80 µg/L. Artemia were allowed to recover from handling for 10 min prior to isotope addition; water samples were obtained from the exposure medium 15 minutes after isotope addition and immediately prior to exposure termination at 24 hours. After 24 hours of exposure, individual artemia were collected from the exposure medium and rinsed three times in isotope-free media to remove ^{75}Se loosely associated with the surface. Individual artemia were blotted dry on paper towels and their wet weight determined to the nearest 100 µg prior to ^{75}Se radioactivity determination. These experiments were repeated in artificial GSL medium at lower concentrations more closely matching concentrations normally found in natural GSL water (0.3-0.6 µg/L). These experiments were designed to also include a nominal concentration of 1 µg/L to provide direct comparison between the two sets of experiments determining selenium uptake from artificial GSL medium. In addition, to these experiments, a set of experiments employing nominal selenium concentrations ranging from 0.3 to 1.0 µg/L were performed in natural GSL water (2336 mOsm; collected). For these experiments with natural GSL medium in which background levels of selenium were expected, water samples from the isotope uptake experiments were verified by analysis of total selenium by Frontier Geosciences, Inc. and measured total selenium concentrations were included in the calculations of selenium uptake.

Determination of dietary selenium intake and subsequent selenium assimilation efficiency in artemia fed a diet of selenium-loaded algae cells (objective 3).

D. viridis cultured in presence of different selenium concentrations served as the dietary source of selenium for artemia. *D. viridis* was cultured under constant light at 18ºC in artificial GSL media (Table 1) in gently aerated Erlenmeyer flasks and media selenium concentrations were monitored daily and adjusted as necessary by addition of ^{75}Se stock solutions or selenium-free media to elevate or reduce media selenium concentrations, respectively. The time required to reach steady state selenium concentrations in *D. viridis* was determined in an initial 40-day experiment and subsequent exposures were 21 days in duration. In addition to daily monitoring of media selenium concentrations, algal density and algal selenium concentrations were determined in algal cells sampled from the cultures and rinsed in ^{75}Se free medium prior to ^{75}Se detection.

D. viridis was harvested for artemia feeding studies at day 20-21 of exposure at which point steady state was achieved. Algae raised at four different media selenium concentrations ranging from 1.2 to 60.4 µg Se/L were used in the present study. Algae were isolated by centrifugation in a microcentrifuge at 8000 rpm which leaves the cells intact after which the radioactive supernatant (algae media) was discarded and cells were rinsed by resuspension in selenium-free media followed by additional centrifugation and media replacement. Radioactivity in the cleansed algal preparations was measured, and algal density in this concentrated cell suspension was determined using a Bright-Line Hemacytometer (Hausser Scientific, Horsham PA) for direct counting using a light microscope.

For each algae selenium concentration, a total of 20 adult artemia were placed in 4 L of GSL medium in a 5-L plastic beaker gently aerated to ensure oxygen saturation and continuous mixing and suspension of algal cells. A total of $37 \cdot 10^6$ cells/L were added to the 4 L of GSL media and artemia were allowed to feed for 60 minutes after which they were removed, rinsed and transferred alive to a gamma counting vial containing 3 ml GSL media. This protocol was chosen from initial experiments to prevent depletion of algal cell density during the feeding experiments and to allow for accurate determination of ingestion rates. Initial experiments monitoring dietary selenium ingestion during a 90-minute period revealed a gut passage time of around 60 minutes at a cell density of approximately $20 \cdot 10^6$ cells/L (Fig. 4), and subsequent feeding experiments were restricted to this duration. Note that linear accumulation of dietary selenium during short term exposure (min), as seen in Fig 4, demonstrates that all ingested selenium is retained in the organism. Plateau of this accumulation curves seen after 60 min means that fecal selenium is being lost at a rate comparable to the rate of ingestion. Gamma counting of individual artemia was conducted immediately and then individual artemia were transferred to 15 ml falcon tubes containing 10 ml of selenium-free GSL medium. The artemia were subsequently fed a non-radioactive algae diet to allow for depuration of unassimilated food overnight

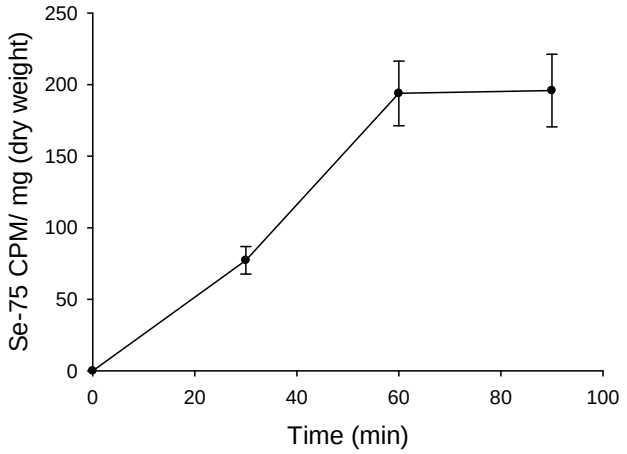

Fig. 4. Selenium accumulation in individual artemia during a 90-minute feeding trail during which artemia were fed *D. viridis* at a cell density of about 20 million cells/L.

after which individual artemia were rinsed and transferred to fresh gamma counting vials containing 3 ml of GSL media in preparation for a second gamma counting. Following this second gamma radioactivity determination, artemia wet weight was determined as above.

Fecal matter from the 15 ml falcon tubes was collected and its ^{75}Se content was determined via gamma counting.

Assimilation efficiency was determined as the ratio of assimilated ^{75}Se to ingested ^{75}Se. The ingestion rate of individual artemia in these studies was determined from the ^{75}Se accumulated during the 60 minutes of feeding and the corresponding selenium concentration in algal cells.

Determination of selenium elimination rates from artemia following selenium accumulation from elevated ambient concentrations (objective 4).

Selenium elimination rate constants were determined for artemia exposed to waterborne and dietary selenium. For the waterborne exposure a total of 30 adult artemia were exposed to 72 µg Se/l for 48 hours without feeding while dietary selenium accumulation in 20 adult artemia was ensured by a 1 hour exposure to ^{75}Se-containing algae. Following the initial exposure individual artemia were rinsed (3 times for the waterborne exposure and once for the dietary exposure) and placed in 3 ml GSL medium in individual gamma counting vials for ^{75}Se determination. After ^{75}Se counting, artemia were placed in 50-ml falcon tubes containing 30 ml GSL medium each and were fed daily. Following this

initial ^{75}Se determination, measurements were performed on a regular basis for a minimum of 20 days allowing for more than 50% depuration of the initial ^{75}Se levels.

Investigate possible regulation of selenium accumulation in artemia during prolonged exposure to selenium (objective 7).

The potential influence of prolonged exposure on selenium accumulation from the water and on assimilation efficiency for dietary exposures was evaluated. To examine uptake rates after long-term selenium exposure a group of adult artemia were exposed to 2.87 ± 0.14 µg Se/L for 14 days. To avoid ^{75}Se accumulation in this group of selenium pre-exposed artemia, these organisms were exposed to non-radioactive selenium. A parallel group of artemia was exposed to identical conditions using radio-labeled ^{75}Se in the water to allow for measurements of selenium concentrations during the 14 days of exposure. Exposure concentrations were adjusted in both these groups of artemia according to ^{75}Se measurements in the radio-labeled group. The ^{75}Se group acted simply as a parallel surrogate to the non-radioactive 14-day exposure to ensure constant and characterized exposure concentrations. In addition to these two groups of artemia, a third group was maintained under control conditions without selenium added. All three groups consisted of 20 adults maintained in 1L gently aerated GSL media (100 g/L) and were fed *D. viridis* daily 3-4 hours prior to adjustments of Se exposure concentrations.

After 14 days of exposure, uptake rates from ^{75}Se-containing GSL medium were determined for the controls and for the artemia exposed to non-radioactive selenium.

These ^{75}Se uptake rate experiments were performed at 2.55 ± 0.11 µg Se/L according to procedures outlined for waterborne experiments elsewhere.

To examine the potential influence of prolonged exposure to dietary selenium on subsequent assimilation efficiencies, *D. viridis* were raised in the presence (resulting in 2.68 µg Se/g dry weight) and absence of selenium. Two algae cultures were raised in presence of selenium, one in which ^{75}Se was employed and one containing the same concentration of non-radioactive selenium. The culture medium selenium concentrations were adjusted in parallel in the two cultures based on measurements of ^{75}Se in the radioactive medium to ensure constant exposure conditions. In parallel with these two selenium-containing cultures, a selenium-free control algae culture was raised simultaneously. All algae cultures were maintained for a minimum of 20 days to ensure steady state selenium concentrations.

Two groups of 30 adult artemia were maintained in 1 L gently aerated GSL (100 g/L) and were fed daily with algae raised in presence or absence of unlabelled selenium (not ^{75}Se). During the 14 days of exposure, exposure beakers were siphoned daily and water was replaced twice weekly.

After these 14 days of exposure to either control or non-radioactive selenium loaded algae (2.68 µg Se/g dry weight), 25 individuals from each group were transferred to 4 L of GSL media and ingestion rate as well as assimilation efficiency were determined as above using a ^{75}Se labeled algae culture (3.73 µg Se/g dry weight).

Verification of the isotope dilution technique

A subset of water and selenium stock solution samples was submitted to Frontier Geosciences, Inc. for total selenium concentration measurements to seek independent analytical verification of the isotope dilution technique. Water samples subjected to total selenium analysis included samples representing all exposure concentrations from the second replicate of the selenium water-borne uptake kinetic experiments with artemia, samples of natural GSL medium used in the parallel selenium uptake experiments as well as a subsample of one of our stock solutions and a commercially available certified stock solution employed in the Grosell laboratory at University of Miami as reference material. Because Frontier Geosciences, Inc. are not licensed to handle radioactive samples, parallel radioactive and non-radioactive stock solutions were made up side by side and both were spiked into GSL media. Total selenium concentrations were determined in both radioactive and non-radioactive stock solutions at University of Miami (Graphite Furnace Atomic Absorption) and both sets of stock solutions were spiked into GSL media. Media spiked with the radioactive stock solutions were used for measurements of selenium uptake in artemia and exposure concentrations were verified using the isotope dilution method. Media spiked with non-radioactive stock solutions were treated similarly to the media spiked with radioactive stock solutions and the predicted selenium concentrations in these solutions were calculated from the measured concentration in the non-radioactive stock solution and the amount added to the GSL medium (Graphite Furnace Atomic Absorption). These non-radioactive GSL media samples were forwarded to Frontier Geosciences, Inc. for selenium analysis.

Results

Evaluation of the isotope dilution technique

Near perfect agreement between selenium measurements performed in the Grosell laboratory at the University of Miami (Graphite Furnace Atomic Absorption) and Frontier Geosciences is evident from Table 2. Furthermore, these two sets of total selenium measurements are in excellent agreement with the results from the isotope dilution technique with the exception of the natural GSL samples. The discrepancy between the isotope dilution technique measurements and the total selenium measurements for these natural GSL samples was expected and reflects selenium concentrations in the GSL medium (not added as part of the University of Miami experiments). The difference between the two sets of measurements range from 0.39 to 0.55 µg Se/L which is in good agreement with commonly occurring concentrations in natural GSL water (Brad Marden's report).

Sample	Isotope dilution	Graphite furnace	Frontier Geosciences
0.3 µg/L, 100 g/L GSL	0.32 (#)	0.32 (*)	0.313
0.6 µg/L, 100 g/L GSL	0.64 (#)	0.65 (*)	0.598
1.0 µg/L, 100 g/L GSL	1.02 (#)	1.08 (*)	1.01
0.3 µg/L, natural GSL	0.33 (#,**)	0.32 (*)	0.868
0.6 µg/L, natural GSL	0.65 (#,**)	0.65 (*)	1.15
1.0 µg/L, natural GSL	1.14 (#,**)	1.08 (*)	1.53
Diluted cold Se stock	N/A	1.35	1.23
Diluted certified Se stock	N/A	0.96	0.923

Table 2. *Measured selenium concentrations in various aqueous media by the isotope dilution technique and the graphite furnace method at the University of Miami compared to values reported by Frontier Geosciences, Inc. (as measured by ICP-MS) for the same samples. * denotes calculated values based on measured Se concentration in "diluted*

*cold Se stock", ** these values do not account for selenium found in natural GSL water prior to experiments. # denotes concentrations determined by the isotope dilution method in samples from actual experiments measuring selenium accumulation in artemia from the water.*

Effects of salinity

To examine the influence of salinity on Se uptake, we exposed *Artemia* to Se under relatively low (100 ppt) and high (160) GSL media salinities for 24H. These values bracket the recorded salinities from GSL. As predicted, selenium uptake from the water was reduced at 160 ppt compared to 100 ppt (Fig 5). However, in contrast to expectations, elevated salinity (160 ppt) resulted in an apparent reduced feeding rate compared to that seen at 100 ppt (Fig 6). Thus, all subsequent experiments were performed at 100 ppt.

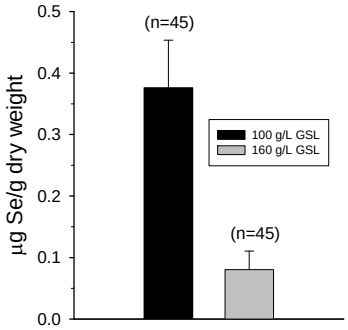

Fig. 5. Influence of salinity on ^{75}Se uptake in adult artemia during a 24-hour exposure to selenium in GSL medium

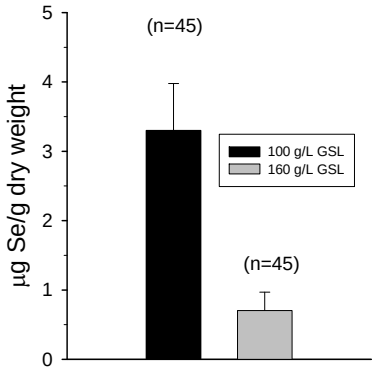

Fig. 6. Average feeding rates determined in three separate experiments from the depletion of algal cells evident from change in absorbance at 750 nm (cell density)

Selenium uptake by artemia from the water

A general trend of increasing selenium uptake rates with increasing ambient selenium concentrations was observed in experiments exposing adult artemia to a range of selenium concentration in GSL media for 24-hour periods (Fig. 7). Upon closer examination, however, an uptake-kinetics saturation pattern is observed for selenium concentrations below 10 µg/L after which selenium uptake rates appear to increase in

proportion to ambient concentrations at 20 and 40 µg/L. We mathematically describe the saturation kinetics by the equation:

Se ug/g dry weight = $((660.2 \cdot C_w)/(1.20+C_w))$

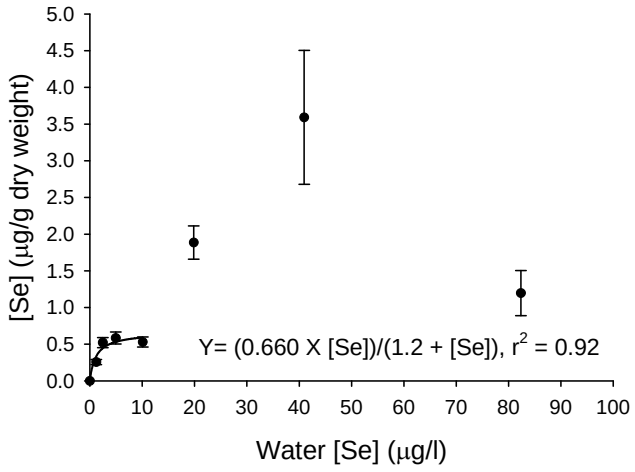

Fig. 7. Selenium accumulation in individual adult artemia as a function of ambient selenium concentrations following a 24-hour exposure. Note the apparent saturation kinetics at concentrations below 10 µg Se/L and the clear elevated increase in uptake at concentrations above 10 µg Se/L identifying the "knee" to be between 10 and 20 µg Se/L.

ku value is surprisingly high relative to other values reported from pelagic saltwater crustaceans (0.024 to 0.027) in the literature[36,43], but is as one would expect lower than that reported from the freshwater cladoceran Daphnia magna (0.187-2.74)[45]. Replication of these measurements using lower selenium concentrations also revealed linear selenium uptake with increasing ambient selenium

At an extreme Se concentration (~80 ug/l), a tendency for reduced selenium accumulation was observed. A near-perfect linear fit describes selenium uptake at ambient selenium concentrations below 2.5 µg/L equivalent of a K_u of 0.211 L/g dry weight/day. This

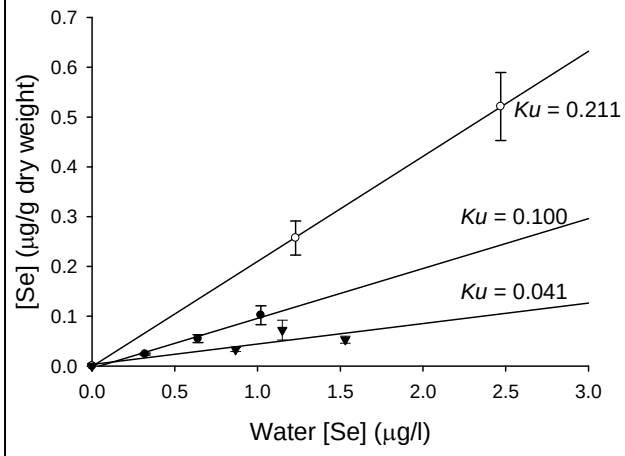

Fig. 8. Selenium uptake by artemia from the water. Comparison of a subset of data presented in Fig. 7. focusing on low ambient selenium concentrations (open symbols) to the second replicate experiments in artificial GSL medium employing more environmentally realistic selenium concentration (filled circles). Selenium accumulation in artemia exposed to similar selenium concentrations in natural GSL water (filled triangles) is lower than corresponding uptake in artificial medium.

concentrations below 1.02 µg/L equivalent to a slightly lower k_u of 0.100 L/g dry weight/day (Fig 8). Furthermore, experiments performed in natural GSL water revealed a lower k_u of 0.041 L/g dry weight/day.

Selenium accumulation in Dunaliella viridis exposed to elevated media selenium.

An unexpected tri-phasic pattern of selenium accumulation in *D. viridis* was observed during an initial 40-day exposure to 2.17 µg Se/L characterized by an initial rapid increase in algal selenium concentrations followed by apparent depuration and subsequent stabilization (Fig. 9). Steady-state selenium concentrations in *D. viridis* appear to be reached in approximately 20 days and subsequent algae selenium loading experiments were performed over this exposure period.

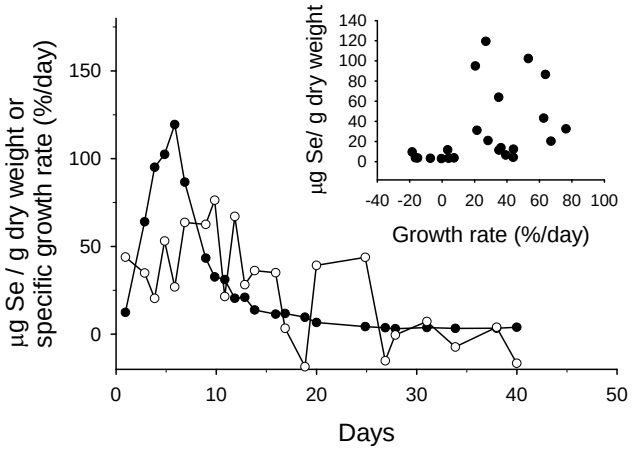

Fig. 9. Selenium concentration in *D. viridis* (dark circles) and specific growth rate (open circles) during 40 days of culturing in the presence of 2.17 µg Se/L. **Insert**: Selenium concentration in algae as a function of specific growth rate

An additional four experiments employing different media selenium concentrations were performed at concentrations ranging from an average 1.2 to 60 µg Se/L and all exhibited a similar pattern of fast initial accumulation followed by depuration and stabilization (Fig. 10). Our radio-isotopic approach allowing for rapid detection of selenium

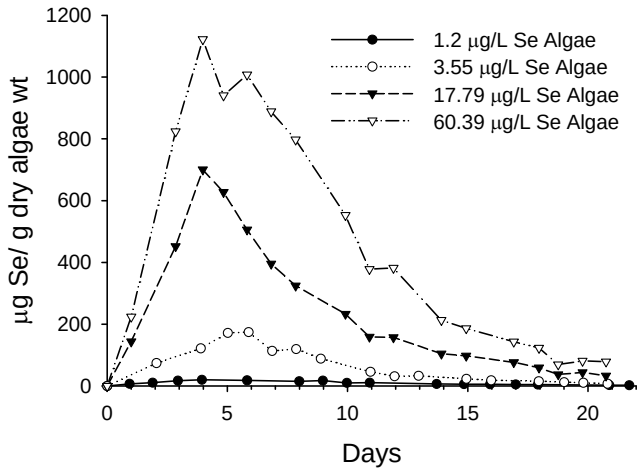

Fig. 10. Time course of selenium accumulation in *D. viridis* raised at four different selenium concentrations. Average media selenium concentrations are indicated on the figure and measured media selenium concentrations throughout the 21 days of culturing are reported in Fig. 10.

concentrations in the exposure media and prompt adjustments ensured relatively stable exposure concentrations during the 20 days of culturing (Fig. 11). Considering the algae selenium concentrations after 21 days of culturing, a less than linear increase in cell selenium concentrations as a function of ambient selenium was observed pointing to lower bioconcentration factors at higher ambient concentrations (Fig. 12).

Comparing the growth rates (as indicated by absorbance at 750 nm throughout 21 days) of algae cultures at different selenium concentrations to growth rates in absence of added selenium revealed highest growth rate at ~18 µg/L (Fig. 13). The lowest growth rate was observed in absence of added selenium and it

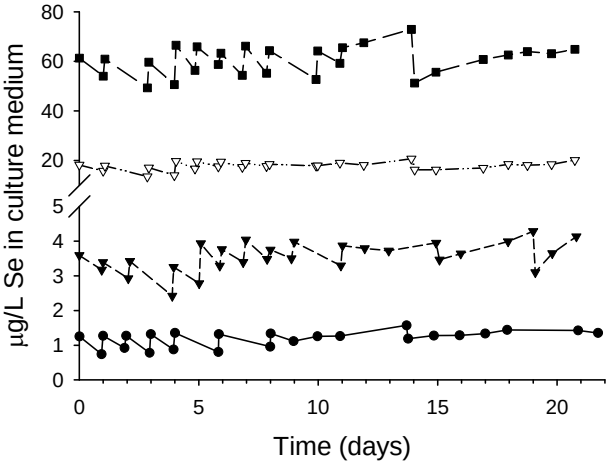

Fig. 11. Media selenium concentrations during 21-day culturing of *D. viridis*. The near-daily fluctuations represent uptake or excretion from the algae cells and corresponding adjustments of exposure concentrations by addition of selenium stock solution or selenium-free culture medium as appropriate.

appeared that the highest employed selenium concentration (60 µg/L) tended to reduce growth of *D. viridis* somewhat (Fig. 14).

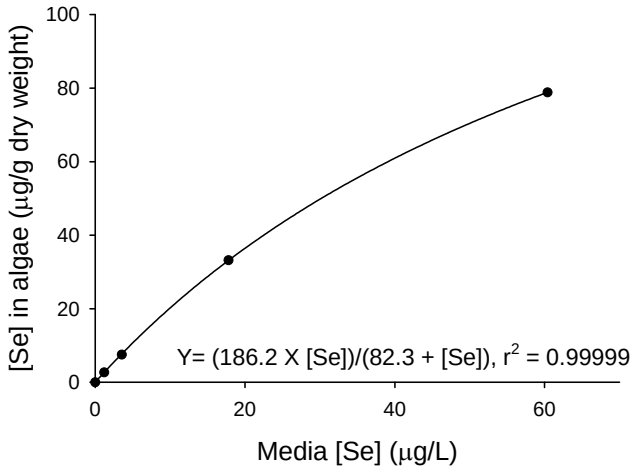

Fig. 12. Steady state selenium concentrations in *D. viridis* after 21 days of exposure as a function of media selenium concentrations

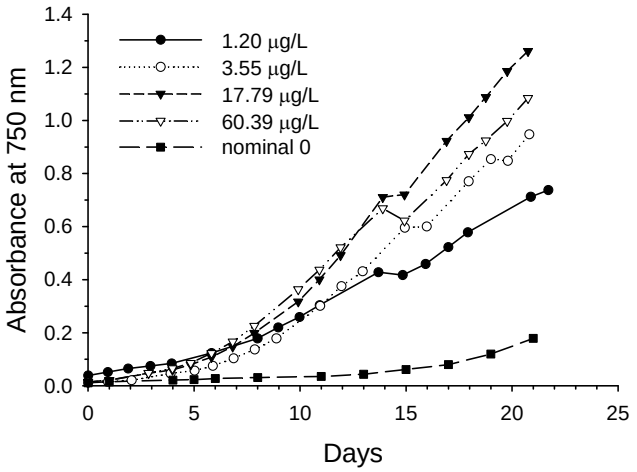

Fig. 13. *D. viridis* growth in culture media of different selenium concentrations.

D. viridis ingestion rate, dietary selenium intake and assimilation efficiency in artemia

The 1-hour feeding experiments revealed feeding rates of 0.185 g/g (dry weight)/day and demonstrated increasing selenium ingestion and assimilation with increasing algae selenium concentrations (Fig. 14). Selenium assimilation efficiency showed a 2nd order exponential decay relationship with a minimum assimilation efficiency of 74% at higher dietary selenium concentrations and a near 100% at low selenium concentrations (Fig. 15 and 16).

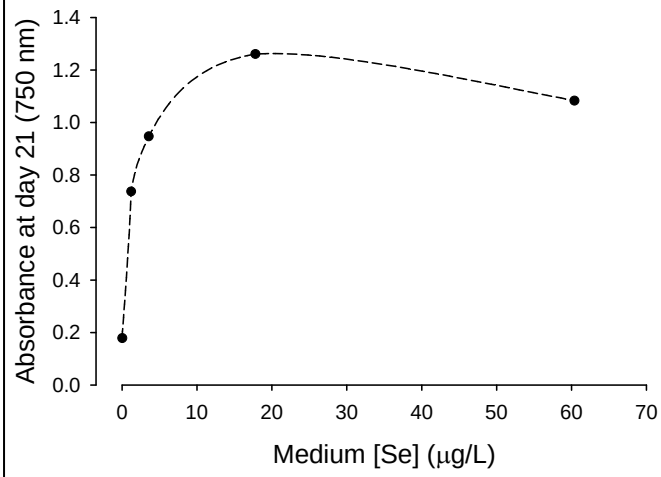

Fig. 14. Cell density after 21 days of culturing at different selenium concentrations.

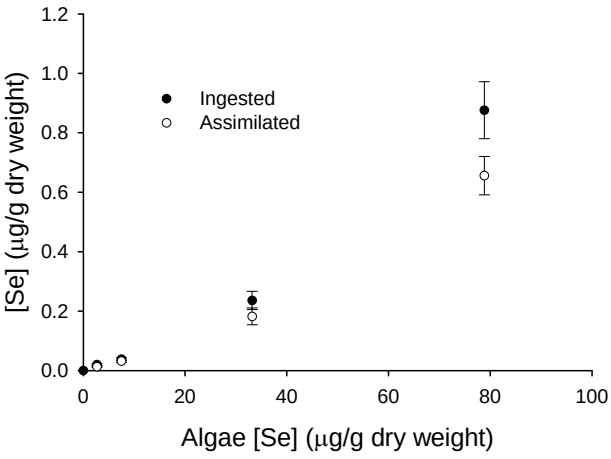

Fig. 15. Ingested (dark symbols) and assimilated (open symbols) selenium in individual artemia following a 60-minute feeding at four different algae selenium concentrations

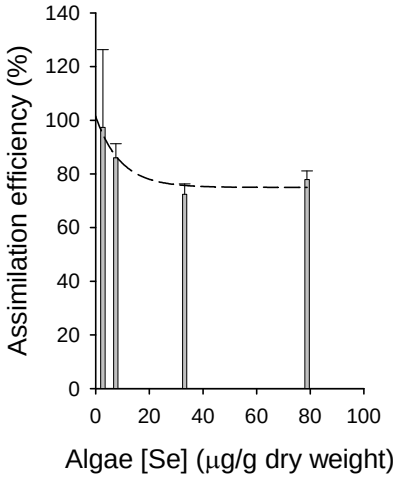

Fig. 16. Assimilation efficiencies for four different algae selenium concentrations calculated from the observations presented in Fig. 14. Data adhere to an exponential decay equation (see text for details), $r^2 = 0.95$.

Selenium elimination rates constants

The possibility of distinct elimination rates for selenium accumulated from the water and the diet was considered. For waterborne selenium, an initial rapid elimination was observed during the first 24 hours following termination of exposure. From day 1 and onward, a simple exponential decay equation describes selenium concentrations in artemia well (r^2=0.99) with a 6.79 % daily selenium loss (Fig. 17). The dietary selenium elimination

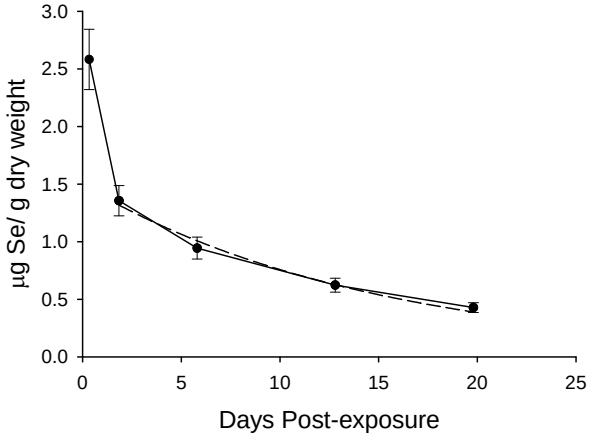

Fig. 17. Elimination of selenium from adult artemia exposed to waterborne selenium (72 µg Se/L) for 48 hours. An elimination rate constant of 0.0679 was obtained from the fitted curve, r^2=0.99.

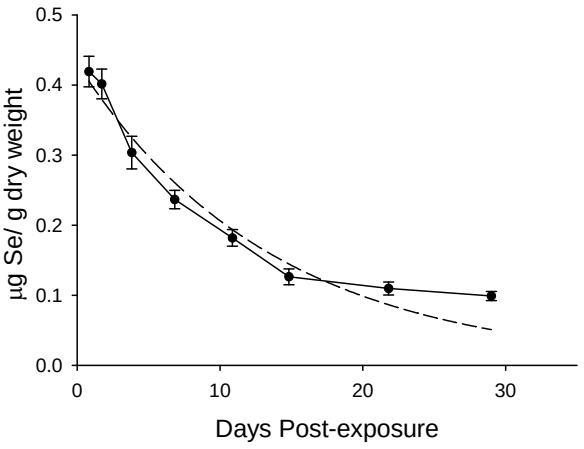

Fig. 18. Elimination of selenium from adult artemia exposed to dietary selenium for 24 hours. An elimination rate constant of 0.0737 was obtained from the fitted curve, $r^2 = 0.95$.

rates of 7.37 % per day remained constant at least during the first 14 days of depuration after which an apparent reduction in elimination rates is evident (Fig.18). The elimination rates were determined from the fitted exponential decay curves (doted lines). Despite initial accumulated selenium concentrations approximately 6-fold higher in waterborne compared to dietary exposures, elimination rates appeared slightly lower for waterborne selenium.

Influence of prolonged exposure on selenium uptake rates.

Two weeks of exposure to elevated, yet environmentally relevant, selenium concentrations tended to reduce selenium uptake rates from the water and assimilation efficiency from the diet. Waterborne exposure to selenium resulted in an apparent 23% reduction in subsequent ^{75}Se labeled selenium uptake although this difference escapes statistical significance (Fig. 19). Similarly, prolonged exposure to dietary selenium concentrations of

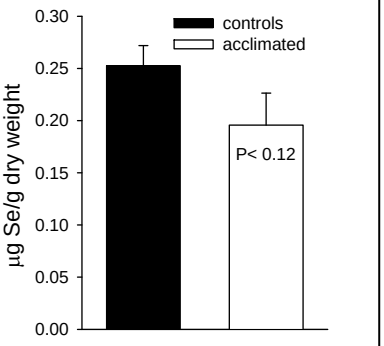

Fig. 19. Selenium uptake from the water at 2.55 ± 0.11 µg Se/L in control artemia and artemia acclimated to 2.87 ± 0.14 µg Se/L for 14 days.

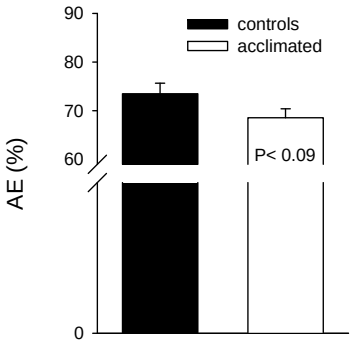

Fig. 20. Assimilation efficiency (AE) from algae containing 3.73 µg Se/g dry weight in control artemia and in artemia acclimated to dietary selenium at 2.68 µg Se/g dry weight for 14 days.

environmental relevance resulted in a modest (not statistically significant) reduction in subsequent assimilation efficiency from 73.5 to 68.6% equivalent to a 7% reduction in total dietary selenium assimilation (Fig. 20).

Discussion

Independent analytical verification by Frontier Geosciences, Inc. confirms the utility of the isotope dilution technique and further demonstrates that background selenium concentrations in media used at the University of Miami are below detection as measured total selenium never exceeds concentrations calculated using the isotope dilution method. The isotope dilution technique continues to be a cost effective, fast and reliable method for determining exposure concentrations in solutions with matrix interference potential and for measuring low concentrations in samples of limited mass. For context, the present project involved the analysis of more than 2400 samples, the majority of which were on the order of 5 mg or less (individual adult artemia).

Initial experiments revealed that 24 hours of exposure to waterborne selenium resulted in linear accumulation in artemia and revealed that exposure concentrations remained constant during this period. Furthermore, it was revealed that 60 minutes of duration for feeding experiments is appropriate for determination of ingestion rates and quantification of selenium ingestion and subsequent assimilation efficiency.

No mortality was observed during selenium uptake experiments and less than 10% mortality was observed in depuration experiments in which repeated handling of individual artemia likely was the cause of mortality.

Influence of salinity

In agreement with expectations, increased GSL salinity from 100 to 160 ppt resulted in a significant reduction in selenium uptake by artemia. Although it is unknown which component(s) of the ionic matrix in the GSL medium is responsible for this observation it appears likely that sulfate is the anion competing with selenium uptake, especially since the artificial GSL medium did not contain phosphate. Early studies demonstrated a direct antagonistic relation between sulfate and selenium uptake in plants[25] and several subsequent studies have revealed that elevated sulfate protects against acute selenium toxicity in algae as well as aquatic organisms, including artemia in freshwater and hypersaline environments[7,14,37].

In contrast, elevated dietary selenium intake (feeding rate) was expected at 160 compared to 100 ppt. Elevated salinity can be expected to be associated with an increased metabolic demand from osmoregulatory processes and such an elevated metabolic cost was expected to be associated with higher feeding rates and thus higher dietary selenium intake in artemia fed *ad lib*. While the reason for apparently reduced selenium ingestion at higher salinities is unknown, the brief duration of the feeding experiments allows for the conclusion that feeding rate is lower at 160 ppt compared to 100 ppt and that ingested/assimilated selenium levels likely are not influenced directly by ambient sulfate levels that might be ingested with food.

Selenium uptake from the water

Selenium uptake from the water displayed a complex pattern of saturation kinetics at concentrations below 10 µg Se/L followed by a sharp increase in selenium uptake rates with a threshold somewhere between 10 and 20 µg Se/L. In addition it appears that selenium uptake is down-regulated at concentrations above 40 µg Se/L, although this later observation is based on a single high selenium concentration. In the following, "high affinity, low capacity system" will refer to the selenium uptake at concentrations below 10 µg Se/L and "low affinity, high capacity system" will refer to the uptake pathways dominating at higher concentrations.

The apparent saturation pattern at relatively low selenium concentrations indicates that selenium is taken up from the water, presumably via the respiratory surface, via protein carriers in epithelial cells. Saturation uptake patterns have also recently been reported for freshwater algae exposed to selenate[15]. Although it seems that high sulfate concentrations may interfere with selenium uptake, the specificity of this putative selenium uptake system is not known but transporters with high specificity for selenium are known from mammalian systems and from plants[4,32,38]. The apparent affinity constant for the high affinity, low capacity selenium uptake system (K_m) which denotes the ambient concentration at which the transport system is half saturated is 1.2 µg Se/L. The significance of this becomes clear when one considers the range of selenium concentrations normally observed in GSL (0.297 to 0.899 µg Se/L, Brad Marden report). Regardless of the nature of the selenium transporters responsible for this high affinity transport system, variations in ambient selenium concentrations within the range

normally observed in GSL will greatly influence the selenium uptake rates by this transport system.

The low affinity, high capacity system dominates at selenium concentrations exceeding those observed in open GSL waters and thus are not a factor for steady state selenium concentrations in GSL artemia.

A situation of an apparent dual carrier uptake system with distinct transport characteristics is not unprecedented and has been observed for copper in the freshwater rainbow trout[20]. Like selenium, copper is an essential micronutrient that is potentially highly toxic and therefore it is not surprising that these two elements might share this unusual uptake pattern.

The apparent reduction in selenium uptake at the highest concentration tested could be a consequence of down regulation of the low affinity, high capacity selenium uptake system but the highest selenium concentration tested is orders of magnitude lower than concentrations considered to be acutely toxic to artemia[14]. Furthermore, the highest tested selenium concentration falls well above concentrations relevant for GSL and uncertainty associated with the reason for apparent reduced uptake at this concentration is of no consequence for predictions on steady state selenium concentrations in GSL artemia.

Selenium accumulation in D. viridis

The careful characterization of selenium accumulation on *D. viridis* for the purpose of providing a natural diet for the study of dietary selenium uptake in artemia under conditions relevant to GSL revealed a complicated pattern of selenium accumulation. An initial increase in cellular selenium concentration in algae cultured in presence of selenium was expected but the clear depuration of cellular selenium concentrations from algae cells despite continued exposure to constant ambient selenium concentrations was not anticipated. An obvious possible explanation for this pattern, growth dilution, can be dismissed based on continued low cellular selenium concentrations during the last 20 days of the 40-day culture period during which cell density remained relatively constant. During this period, net growth was minimal but no increase in cellular selenium concentrations was observed and values remained much below peak concentrations observed around day 5-8 of culturing. Furthermore, calculations of specific growth rate in the algae culture (daily % increase in cell density) revealed that growth rates were also high during the initial rapid accumulation phase observed during the first week or so of culture. A final observation of lack of correlation between algal cellular selenium concentrations and specific growth rate also argues against growth dilution as an explanation for the observed selenium depuration during continued exposure.

Two possible explanations remain that may account for the observed reduction in cellular selenium concentrations during continued exposure. For one, reduced selenium uptake as a negative feedback to elevated cellular selenium concentrations combined with constant growth and selenium elimination would result in reduced cellular selenium concentrations. A second possibility is that selenium elimination is stimulated by elevated

cellular selenium concentrations which, even when combined with constant uptake, would result in reduced cellular selenium concentrations. Obviously, a combination of reduced uptake and stimulated excretion cannot be dismissed as a possibility. Indeed, selenium biotransformation by salinity tolerant phytoplankton has been described to include the formation of volatile alkylselenides which may account for the apparent selenium excretion[13].

While activation of a selenium export system is the only way to account for selenium excretion, reduced uptake could potentially be accounted for by a down regulation (reduction in numbers) of selenium uptake proteins or be explained by cellular excretion of substances rendering ambient selenium less available for cellular uptake. This latter explanation could be highly important in algal culture situations where cell densities are extremely high compared to natural situations but might be less important under natural conditions. In contrast, a down regulation of selenium uptake proteins would have the same effect in algal cultures as in natural algae populations.
In any case, the employed long-term exposures of algae in the present study ensures that algal biotransformation of selenium to organic forms which is significant for dietary selenium availability occurred.

The bioconcentration factors for *D. viridis* at steady state were $2.23 \cdot 10^3$, $2.16 \cdot 10^3$, $1.87 \cdot 10^3$ and $1.31 \cdot 10^3$ at 1.2, 3.6, 17.8 and 60.4 µg Se/L, respectively (calculated from the data in Fig 12) and thus adhere to what appears to be a general pattern of reduced bioconcentration factors with increasing exposure concentrations[31]. An important

consequence of the dynamic Se accumulation pattern over time in *D. viridis* is that bioconcentration factors will differ depending on what point in time during exposure the algal selenium concentrations are considered. The above bioconcentration factors, although somewhat higher, compare favorably to an overall bioconcentration factor for seston in GSL of $0.64 \cdot 10^3$ (based on mean concentrations from 2006, Brad Marden report), although they are somewhat higher. A possible explanation for the higher bioconcentration factors for *D. viridis* under laboratory conditions compared to the field may reflect that seston from GSL is comprised in part of organic material without cellular metabolic activity and thus selenium concentrating processes.

Trophic selenium transfer to artemia

Gut retention time for artemia fed *D. viridis* is 60 minutes and ingestion rates at the cell densities employed for the present study were 0.021 g algae dry weight/day/g artemia wet weight, which is equivalent to 0.185 g algae dry weight/day/g artemia dry weight. This ingestion rate is comparable to feeding rates reported for marine zooplankton including copepods and mysids although slightly lower than the reported range from 0.33 and 0.44 g algae dry weight/day/g artemia dry weight[30].

The assimilation efficiencies determined in the present study were not constant across exposure concentrations. To the best of our knowledge no studies to date have considered the influence of dietary exposure concentrations on selenium assimilation efficiency and it is generally assumed to be constant regardless of concentration for metals[30]. The exponential decay equation describing the relationship between dietary selenium

concentration and assimilation illustrates that selenium assimilation efficiency in artemia fed *D. viridis* ranges from 75% at high concentrations to 100% at very low selenium concentrations. This relationship is in agreement with the saturation pattern observed for uptake of selenium from the water and strongly suggests that intestinal selenium uptake is mediated by specific transport pathways that become limiting for uptake at higher selenium concentrations. The assimilation efficiencies observed in the present study (>75%) compare favorably with earlier reports ranging from 30-86%[29,36,39,44] but cannot be assumed to be constant across exposure concentrations. The assimilation efficiencies determined as part of the present study represent a suspension feeding/algae relationship that is directly relevant to GSL and considers algae in steady state with respect to selenium concentrations. While using algae at steady state represents a realistic situation for chronic exposures, it is unknown how factors like cell density (and thus feeding rate) and seston (rather than pure algae) as a food source might influence dietary selenium assimilation.

Selenium elimination by artemia

Considering first elimination of selenium accumulated from the water, an 80% depuration was obtained during a 20-day period with an initial rapid selenium loss during the first 24 hours following termination of exposure. An elimination rate constant of 6.79%/day was determined from fitted exponential decay curves based on data points collected after the initial rapid depuration phase. Similar observations of rapid initial elimination of metals have been reported previously and are believed to be associated with dissociation of surface-bound metal. The rapid initial elimination phase was not considered when

deriving elimination rate constants because it most likely does not reflect the physiology of organisms chronically exposed in natural environments[8,10,11].

Considering next the elimination of dietary selenium originating from a *D. viridis* diet, a near 80% depuration was also obtained approximately 20 days after ingestion of a ^{75}Se labeled algae diet. The elimination rate constant for dietary selenium was 7.37 ± 0.33%/day and thus tended to be slightly higher than the 6.79 ± 0.34%/day observed for waterborne selenium. From both the waterborne and dietary selenium elimination experiments it appears that elimination rate constants are independent of accumulated selenium concentrations, which is consistent with most earlier studies. Although slightly different, the elimination rate constants observed in the present study for waterborne and dietary selenium are in agreement with elimination rate constants reported for many other invertebrates for a number of different metals[30].

A model to predict steady state selenium concentrations in artemia (Objective 5)

The development of a model to predict steady state selenium concentrations in *artemia* under conditions relevant to GSL was inspired by the DYMBAM model approach[35]. In brief, the differential equations describing this model have been solved to determine selenium concentrations at steady state (constant selenium concentration in the organism, *C*ss) as:

$$Css = [(k_u \cdot C_w) + (AE \cdot IR \cdot C_f)]/(k_e + g)$$

where:

k_u = is the uptake rate constant from water

C_w = waterborne selenium concentration

AE = dietary assimilation efficiency

IR = ingestion rate

C_f = dietary selenium concentration

k_e = elimination rate constant

g = growth dilution

The approach presented in the following deviates slightly from the original DYMBAM model in that in considers the two (slightly) different k_e's, one for waterborne Se (k_ew) and one for dietary Se (k_ef) discussed above.

Thus the principal model developed for the steady state selenium concentrations in brine shrimp in the GSL is as follows:

$$\mathbf{Ss[Se] = ((}k_u \cdot \mathbf{C_w)}/k_e\mathbf{w) + ((AE \cdot IR \cdot C_f)}/k_e\mathbf{f)}$$

Note that growth dilution "g" is omitted from the model since it has been developed for adult artemia.

In addition to this deviation, uptake rate from the water (k_u) is considered in two different ways: in scenario I a traditional k_u is used as in previous reports whereas in scenario II it

is reflected by a Michaelis-Menten kinetics equation (Fig. 21). These two different scenarios result in slightly different predicted steady state selenium concentrations but since only one apply to environmental conditions relevant for GSL, only one set of steady state concentrations are reported. Furthermore, our observations of varying assimilation efficiency (AE) depending on dietary Se concentrations prompted the use of an equation rather than a constant to describe AE.

The constants/equations used for the developed ss[Se] model are listed in Table 3.

	Waterborne		**Dietary**
Parameter	*Scenario I*	*Scenario II*	
K_u	0.211/0.100 (0.156)	$(660.2 \cdot C_w)/(1.20+C_w)$	-
C_w	Input variable		-
k_ew or k_ef	0.0679	0.0679	0.0737
AE	-	-	$(74.97+26.54^{-0.1088Cf}) \cdot 10^{-2}$
IR	-	-	0.185
C_f	-	-	Input variable

Table 3. *Individual model parameters for the ss[Se] model. Uptake parameters ingestion rates are expressed per g dry weight*

Waterborne exposure ***($\underline{(k_u \cdot C_w)/k_e w}$):***

Uptake:

Scenario I **($\underline{k_u \cdot C_w}$):**

The traditional uptake rate constant (k_u) can be determined from the near-linear part of the uptake kinetics curve to be 0.211 l/g dry weight/day) and applies to ambient selenium concentrations < 2.5 µg/L since the uptake kinetics curve is only linear below this concentration (Fig 21). The two replicate experiments revealed slightly different k_u's

(0.211 and 0.100, respectively). For predictions of selenium steady state concentrations a mean k_u of 0.156 as a traditional uptake rate constant has been applied.

Scenario II:

From the Michaelis-Menten saturation kinetics applying to selenium concentrations below 10 µg/L, an uptake rate constant (or equation rather, Fig. 21) was determined to be:

$k_u = (660.2 \cdot C_w)/(1.2 + C_w)$, $r^2 = 0.92$

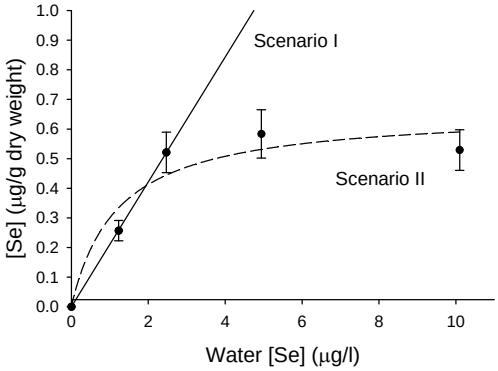

Fig. 21. Subset of data presented in Fig. 7. focusing on low ambient selenium concentrations. A traditional linear K_u applies to exposure concentrations below 2.5 µg Se/L but saturation kinetics apply to a wider range of concentrations from 0 to 10 µg Se/L.

Note that the constant in scenario I is considerably higher than previously reported k_u's for pelagic crustaceans ranging from 0.024-0.027[36,39] but is in good agreement with k_u's for estuarine macroinvertebrates[3] and that it is determined for low selenium concentrations relevant for GSL. The k_u determined for natural GSL water is in closer agreement with previously determined k_u's for pelagic crustaceans. It is unknown why natural GSL water would yield a lower k_u than observed in artificial GSL since the salinity of the natural GSL sample used was lower than that of the artificial medium. Employing scenario II for higher concentrations reveals numbers in closer agreement with the above-mentioned previous values. In contrast, employing scenario II to predict

steady state selenium concentrations at low water-borne selenium (<2 µg/L) results in higher selenium steady state selenium concentrations than scenario I. These higher predicted values for scenario II are a consequence mainly of the curve fitting and are probably unrealistic. Consequently, only model predictions using scenario I are reported in the following.

Elimination: The rate constant of loss (k_e) relevant to selenium accumulated from waterborne exposure are discussed above.

Steady state Se concentrations (ss[Se]) in artemia arising from waterborne exposures in artificial GSL water (the first part of the ss[Se] model above) can be estimated using the following equations:

Scenario I: Waterborne ss[Se] = $((0.156 \cdot C_w)/0.0679)$

Scenario II: Waterborne ss[Se] = $[((660.2 \cdot C_w)/(1.2 + C_w))]/ 0.0679$

Dietary exposure ($(AE \cdot IR \cdot C_f)/k_ef$):

Uptake: The assimilation efficiency (AE) is normally assumed to be constant in DYMBAM models regardless of dietary metal concentration. However, the present project identified that assimilation efficiency decreases with increasing dietary selenium concentrations and that it adheres to an exponential decay equation ($74.97+26.54^{-0.1088 \cdot Sef}$) that is used to predict AE in the present ss[Se] model.

Conversion from artemia wet weight to dry weight

The water content of adult artemia used in the present investigation was 88.6 ± 0.5 % (n=12).

Predicted steady state selenium concentrations (ss[Se])

Table 4 shows artemia steady state selenium concentrations according to the model parameters for scenario I described above for waterborne selenium, combined with the dietary contribution. Scenario I is used since it describes the uptake directly from the water most accurately at low concentrations relevant to GSL. Highlighted values represent mean measured concentrations from GSL (mean selenium concentrations in GSL during the period from April to December 2006; data provided by Brad Marden) and corresponding predicted ss[Se] according to the scenarios described above. Measured total selenium concentrations in artemia from GSL range from 0.5 to 3.3 with an arithmetric mean of 1.185 µg Se/g dry weight and the model prediction of 2.62 µg Se/g dry weight for scenario I and it thus in reasonable agreement. A slightly better agreement is achieved using the k_u obtained from experiments with natural GSL water for which the predicted selenium concentration at steady state is 1.62. Note that for the GSL selenium concentrations observed in April – December 2006, scenario I is the recommended model.

The model predictions and measurements of artemia selenium concentrations reported by Brad Marden are in reasonably good agreement with measured selenium concentrations in artemia collected from GSL in 2002 which range from 2.86 to 3.38 µg Se/g dry weight for artemia collected in open GSL water[5]. However, in the study by Brix and co-workers little if any effect of ambient selenium on artemia selenium concentrations was observed at concentrations below 30 µg Se/L. The "knee" in the accumulation curve appears to be somewhere between 30 and 80 µg Se/L for field-collected artemia[5], which is somewhat higher than the 10-20 µg Se/L observed in the present study. A similar pattern was observed by Brooks in a study for Kennecott Utah Copper, Inc., which reported the "knee" in laboratory studies of selenium-exposed artemia to be around 50 µg Se/L and artemia selenium concentrations of around 2-3 µg Se/g dry weight at concentrations below this threshold. Using a conservative approach, fitting a linear relationship between artemia and water selenium concentrations from field-collected samples Brix and co-workers suggested that 5 mg Se/kg dry weight in artemia would not be reached until ambient selenium concentrations reached 27 µg Se/L[5]. The models developed as part of the present study are not suited to evaluate ambient concentrations as high as 27 µg Se/L and should not be used to consider situations of selenium concentrations above 2.5 and 10 µg Se/L for scenarios I and II, respectively. However, both model scenarios agree that artemia steady-state concentrations of 5 mg/kg will be reached at concentrations considerably below 27 µg Se/L. The reason(s) for this discrepancy is unknown but it is possible that field collected artemia were not at steady state with respect to selenium concentrations due to limited residence time in the local environment sampled.

Interestingly, both field-collected and laboratory-reared artemia display the “knee” in the accumulation curve although at slightly different exposure concentrations. From the present study one can conclude that this shape of the accumulation curve can be ascribed to uptake from the water rather than the diet. This conclusion is based on the proportionality of algae selenium accumulation in relation to media selenium concentrations and artemia algae ingestion rate, which is constant across the tested dietary selenium concentrations. These observations combined cannot account for an observed pattern of selenium uptake at higher selenium concentrations. In contrast, uptake from the water shows an accumulation pattern similar to that observed in GSL collected artemia (although with different thresholds) with an greatly elevated increase in accumulated selenium above the “knee”.

An interesting observation arising from model predictions made possible through the present study is that waterborne selenium uptake contributes significantly to steady state concentrations in GSL artemia. Using the mean selenium concentrations for seston and water collected from GSL above, water contributes 52% of the steady state selenium concentrations (model scenario I). This conclusion is supported by the observation that uptake from the water likely dictates the accumulation pattern with increasing ambient concentration as waterborne uptake displays the hockey stick-shaped patterns observed for selenium accumulation in artemia collected from GSL.

The K_u determined as part of the present study for low ambient selenium concentrations is high compared to previous reports, which likely explains the relatively high waterborne

contribution to steady state selenium concentrations in artemia. However, it should be noted that since uptake from the water and assimilation efficiencies are not strict linear functions of selenium concentrations the relative contribution of the two uptake pathways will depend on environmental conditions and selenium concentrations.

Acclimation – reduced selenium uptake?

Reduced metal uptake and elevated metal excretion has been observed during prolonged exposure to essential elements[17,18,19,21,22,27] and serves to maintain stable tissue levels despite elevated environmental concentrations. Considering the essentiality of selenium, homeostatic control of selenium in artemia is likely and might involve both reduced uptake and elevated elimination. When predicting steady state concentrations using biodynamic models, such physiological responses may go unnoticed and could result in overestimation of steady state concentrations. The approach employed to determine selenium elimination in the present study involved a brief exposure but several weeks of depuration measurements which likely would have captured and included any influence of adjustments to serve homeostatic control. In contrast, the uptake measurements, dietary as well as waterborne, were performed over 1-24 hours using artemia not previously exposed to selenium. The possibility of reduced uptake from the water or reduced dietary selenium assimilation efficiency in artemia following prolonged selenium exposure was therefore examined in the present study. For both waterborne uptake and dietary assimilation efficiency, the predicted reductions following prolonged exposure were observed but were statistically insignificant. Although no statistical significance was noted, both waterborne uptake and dietary assimilation efficiency tended to drop as

predicted but only to a modest extent. The combined effect of these reductions in selenium uptake likely would not exceed a 10-20% reduction in predicted steady state selenium concentrations in artemia. This potential effect on steady state concentrations is not currently represented by the models (scenario I or II) but the models could be adjusted to accommodate for this effect should it be desired.

Conclusions

It appears that, regardless of the route of uptake, selenium accumulation is lower at higher salinities. Although the generality of this observation across a wider range of salinities remains to be demonstrated, it appears that steady-state selenium concentrations, all other factors being equal, may correlate negatively with ambient salinity.

Algae exposure time is of great importance for apparent bioconcentration factors, as algae (at least *D. viridis*) display a complex selenium accumulation pattern over time.

At steady state, *D. viridis* display a negative correlation between selenium bioconcentration factors and exposure concentration.

Homeostatic control of selenium in *D. viridis* is suggested by the reduced cellular selenium concentration during continued exposure, a reduction that cannot be accounted for by growth dilution.

Selenium uptake from the water displays saturation kinetics at low ambient concentrations (<10 µg Se/L) with a high affinity constant and relatively high K_u. At higher concentrations, a low affinity, high capacity uptake system contributed to a "hockey stick-shaped" accumulation pattern.

Selenium assimilation efficiency by artemia is not constant. Near 100% assimilation efficiency applies to low dietary (i.e., *D. viridis*) selenium concentrations while 75% is relevant for higher concentrations.

A developed set of DYMBAM-type models allows for predictions of steady-state selenium concentrations in artemia under conditions relevant to GSL. Model predictions are in good agreement with measured values from GSL and other laboratory studies.

The models ascribe waterborne uptake as a significant contribution to steady state selenium concentrations in artemia.

Acclimation (likely to occur during prolonged exposure) possibly results in a modest reduction of selenium uptake from both waterborne and dietary sources.

Recommendations

The reason for the reduced cellular selenium concentration in *D. viridis* during continued exposure remains unknown and it is uncertain if such a pattern would apply under natural conditions. It is advised that the two possible explanations for reduced selenium uptake

(reduced number of selenium transporters versus excretion of substances rendering selenium less available for uptake) are examined experimentally.

Furthermore, it is desirable to examine if selenium taken up during the early phases of algae growth and accumulation is more or less amendable to trophic transfer to artemia.

The above modeling effort and conclusions are based on experiments performed at a single and high algae cell density and a uniform, single-species algae diet. None of these conditions are completely realistic for GSL. Algae densities are always below the densities employed in the present study and seston rather than pure algae communities are the natural food source for artemia in GSL. A lower cell density might result in a lower feeding rate, which in turn may result in higher assimilation efficiency. Furthermore, seston rather than pure algae diets might reduce assimilation efficiency. The combined influence of these possible factors on dietary selenium uptake is impossible to accurately predict without further studies.

The isotopic approach has proven very effective for fast feedback on exposure concentrations and thus for the maintenance of constant exposure concentrations and for determination of selenium uptake and accumulation in a cost effective manner. In addition, the resolution and sensitivity of isotope measurements is superior to that of other analytical techniques. However, this technique is not without potential drawbacks. From a biodynamic modeling perspective it is assumed that ^{75}Se uptake, internal distribution and subsequent elimination reflect all components of selenium homeostasis

and that they are in equilibrium with internal selenium stores present in the organisms prior to isotope exposure. While this is not a problem for uptake rate measurements from the water or for dietary uptake measurements from a chronically exposed diet as used in the present study, it may influence elimination rate constant determination. The extensive duration of the depuration measurements in the present study were aimed at limiting this potential problem but it is not known for certain if ^{75}Se elimination truly reflects overall selenium elimination. A set of validation experiments comparing DYMBAM model predictions from isotope measurements to actual measured total selenium concentrations in artemia held under identical conditions would address this uncertainty.

Table 4. Scenario I: total ss[Se] (µg Se/g dry weight) in artemia

	Dietary [Se]											
Water borne [Se]	**0**	**0.2**	**0.4**	**0.504**	**0.6**	**0.8**	**1.0**	**1.2**	**1.4**	**1.6**	**1.8**	**2.0**
0	0	0.5	1	1.26	1.50	1.99	2.47	2.95	3.42	3.89	4.35	4.81
0.2	0.46	0.96	1.46	1.72	1.96	2.45	2.93	3.41	3.88	4.35	4.81	5.27
0.4	0.92	1.42	1.92	2.18	2.42	2.91	3.39	3.87	4.34	4.81	5.27	5.73
0.597	1.36	1.87	2.36	2.62	2.86	3.35	3.83	4.31	4.78	5.25	5.72	6.18
0.6	1.38	1.88	2.38	2.64	2.88	3.37	3.85	4.33	4.80	5.27	5.73	6.19
0.8	1.84	2.43	2.84	3.10	3.34	3.83	4.31	4.79	5.26	5.73	6.19	6.65
1.0	2.30	2.86	3.30	3.56	3.80	4.29	4.77	5.25	5.72	6.19	6.65	7.11
1.2	2.76	3.26	3.76	4.02	4.26	4.75	5.23	5.71	6.18	6.65	7.11	7.57
1.4	3.32	3.72	4.22	4.48	4.72	5.20	5.69	6.17	6.64	7.11	7.57	8.03
1.6	3.68	4.18	4.68	4.94	5.18	5.66	6.15	6.63	7.10	7.57	8.03	8.49
1.8	4.14	4.64	5.14	5.40	5.64	6.12	6.61	7.09	7.56	8.03	8.49	8.95
2.0	4.60	5.10	5.60	5.86	6.10	6.58	7.07	7.55	8.02	8.49	8.95	9.41

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STANDARD OPERATING PROCEDURES

Standard Procedures for Se-75 Experiments – Uptake from the Water

(Objectives 1, 2, 6 & 7)

1. Acclimate artemia for a minimum of 48 hours to test media (for example 100 g/L and 160 g/L GSL media) in 1-L tripour beakers containing ~ 800 mls media. Transfer approximately 50 adult artemia from culture tank to acclimation beaker by gently netting with a fine mesh fish net, and feed 1 ml of algae food daily. Brine shrimp food is made by adding 1 g of Wardley Premium Algae Discs per 20 mls of deionized water and blending thoroughly. It is kept refrigerated.
2. Prepare Se-75 stock solution in a 1.5-ml micro-centrifuge tube 24 hours prior to test initiation to allow for complete equilibration. Se-75 stock is made with Se-75 isotope, unlabelled ("cold") Se and DI water in a ratio to provide the desired Specific Activity and volume necessary for test beaker spikes and determination of radioactivity. Keep frozen (-20ºC) until ready for use to inhibit microbial activity.
3. Immediately prior to test initiation, remove 20 artemia from acclimation beaker individually with a plastic transfer pipette and place in 80 mls fresh test media in a 100-ml beaker (this is done to minimize the introduction of fouled water from the acclimation beaker into the test beaker).
4. Add 25 mls of fresh test media to a clean 50-ml beaker.
5. Carefully add 20 of the rinsed individual artemia to the beaker using a plastic transfer pipette, minimizing the amount of liquid transferred with each artemia. This density is similar to the density of the artemia in the main cultures.

6. Wait 10 minutes for the artemia to recover from handling and to acclimate to the test beaker.
7. Spike the test beaker with the appropriate volume of Se-75 stock solution to reach desired concentration. The small volume of the Se spike (i.e. 20 μl) does not significantly alter the water chemistry, including pH of the test beaker.
8. Gently aerate the beaker with capillary tubing to ensure mixing and full air saturation and cover with a glass Petri dish (Figures 1a and 1b).
9. 10 minutes after isotope addition take an initial water sample (100 μl) for determination of Se-75.
10. After 24 hours of exposure take a final water sample (100 μL) for determination of Se-75. Preliminary experiments have shown that in this experimental setup, the amount of radioactivity, and therefore the [Se], remains constant during the 24-hour exposure (Figure 2).
11. Carefully remove individual artemia with plastic transfer pipette and transfer them (individually) through a series of 3 rinses (10-15 mls each) of fresh media in a 6-well plate (Figure 3). This procedure has been tested and has revealed no remaining isotope contamination after the 2nd rinse (Figure 4).
12. After rinsing, carefully blot individuals dry on a paper towel, determine mass on weighing paper to nearest 10 μg, then place into plastic culture tube for gamma counter.
13. Rinse and weigh 10 artemia, take another final water sample (100 μL), then rinse and weigh 5 more artemia for a total of 15 individuals.

14. Dilute the Se-75 stock as appropriate to measure cold Se on the GFAAS; then take three 10-µL samples of this diluted stock to be read on gamma counter.
15. Determine CPMs of all samples on gamma counter: blank, initial water sample, blank, final water sample, 10 individuals, final water sample #2, 5 individuals, and diluted Se-75 stock.
16. Measure cold Se on GFAAS and determine specific activity of Se-75 stock by dividing: (cpm/L) / (µg Se/L) = cpm/µg Se.
17. Calculate Se uptake according to: (cpm/individual) / (cpm/µg) = µg Se/individual.

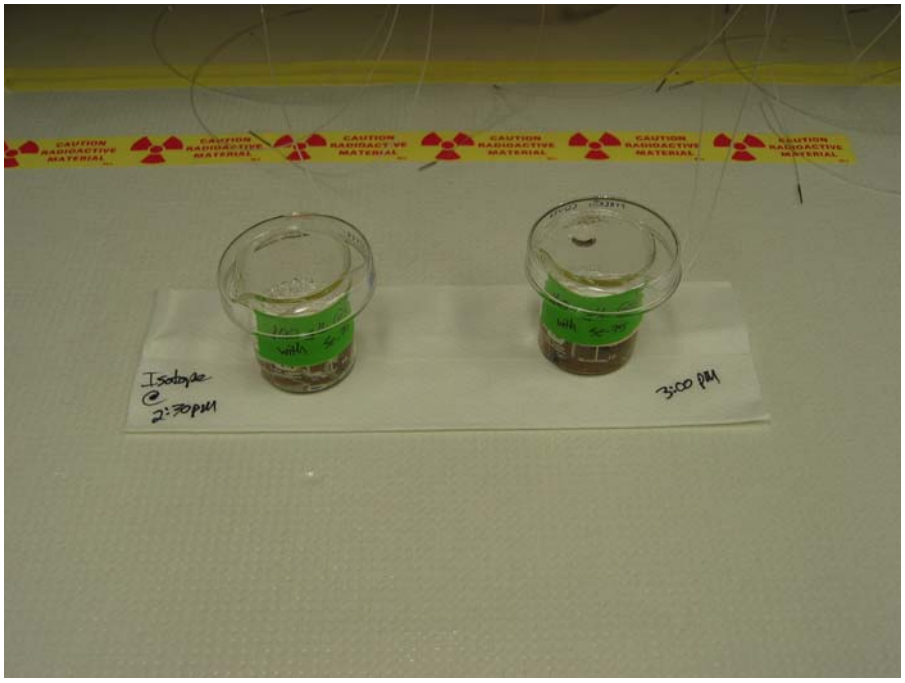

Figure 1a.

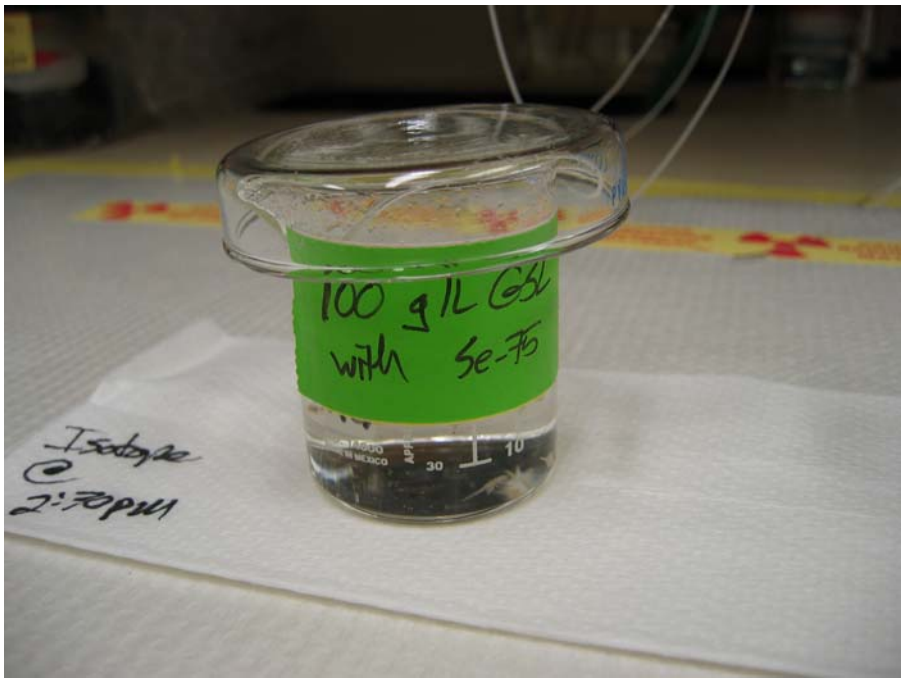

Figure 1b.

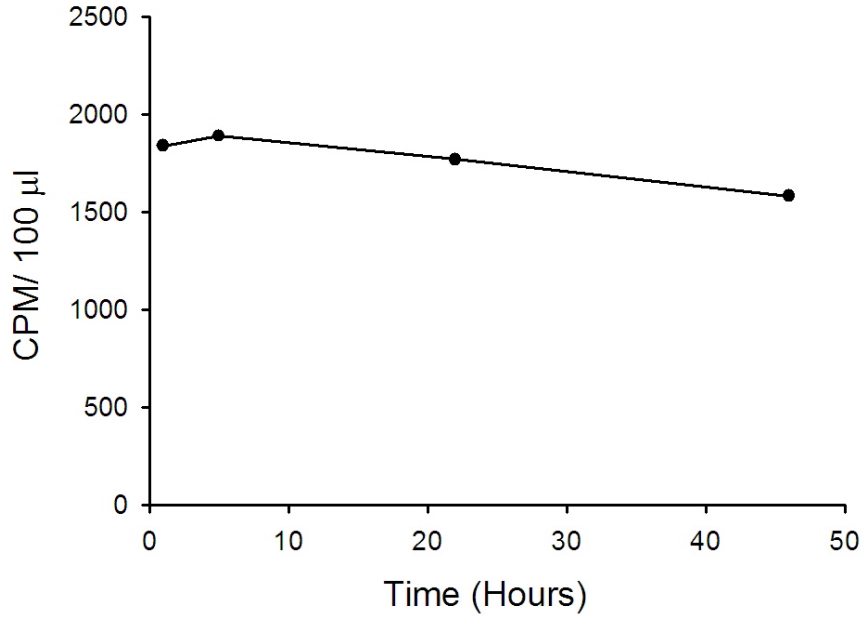

Figure 2. Radioactivity in 100-ml samples of Se-75 exposure water sampled at 1, 5, 22 and 46 hours during preliminary experiments. Radioactivity, and therefore [Se], remains relatively constant over time.

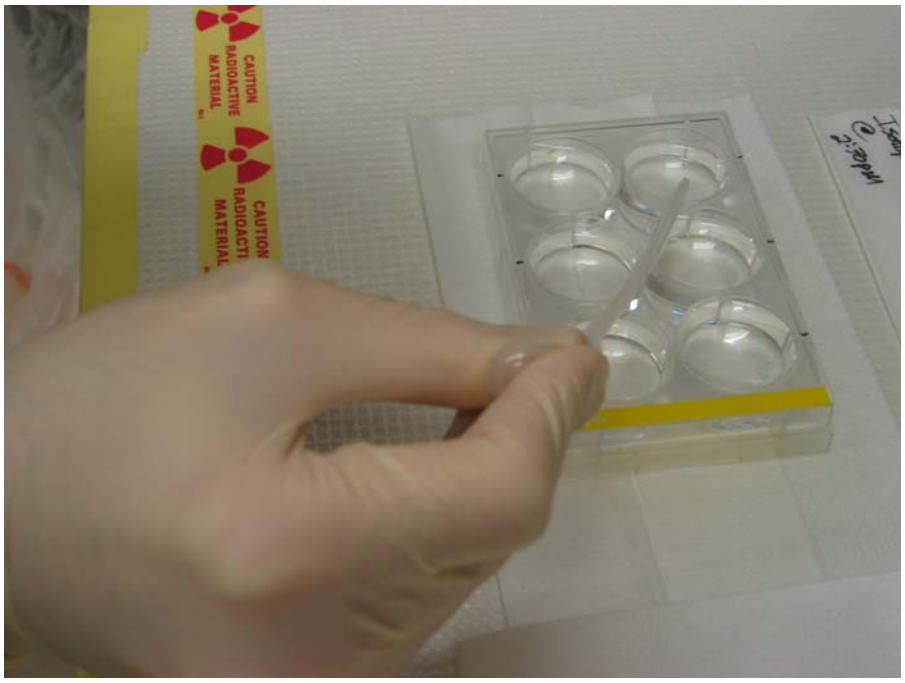

Figure 3.

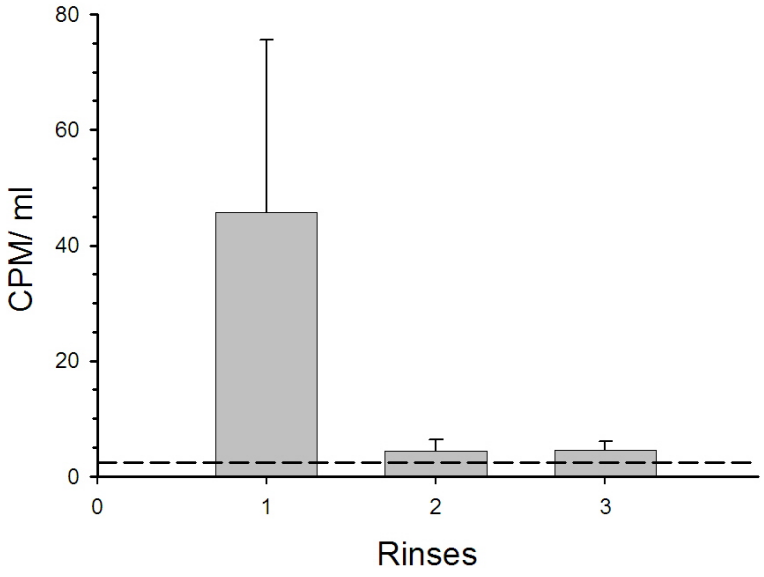

Figure 4. Radioactivity in the three rinse waters. Radioactivity in the second and third rinse waters is not significantly different from background levels (dotted line). For these experiments a total volume of 1 ml was counted for Se-75 activity.

Standard Procedures for Feeding Rate Experiment (Objective 1)

1. Remove ~100 adult, age-matched artemia from main culture tank and rinse in fresh media in a 200-ml beaker. (Artemia from the same hatch date and culture tank are very similar in size.)
2. Add 30 mls of fresh 100 g/L and 160 g/L GSL media to 50 ml centrifuge tubes.
3. Carefully transfer 15 artemia to each tube with a plastic transfer pipette, minimizing the amount of liquid transferred with each artemia.
4. Gently aerate the tube with capillary tubing to ensure even mixing and full air saturation and allow artemia a minimum of 10 min to recover from handling.
5. Add 2 mls of *Dunaliella viridis* concentrate to each tube (save sample of algae to perform cell counts for algae density).
6. Immediately take an initial water sample of 1 ml, and then take 1 ml sample every 10 minutes up to 60 minutes.
7. After thorough mixing to avoid problems with settling, measure the absorbance of all water samples on a spectrophotometer at 750 nm.
8. Plot absorbance over time and perform a linear regression on the decrease in absorbance to obtain the slope (change in absorbance per minute).
9. Divide slope by number of individuals per tube and express feeding rate as change in absorbance/ minute/ individual.

Standard Procedures for Determination of Dietary Selenium Intake

1. Grow *Dunaliella viridis* in algae media as outlined in the Scope of Work for 20 days under constant Se-75 labeled selenium concentrations (nominal 3, 15 and 50 µg/l)
2. Remove adult, size-matched *Artemia* from main culture tank and rinse in fresh media in a 200-ml beaker. (*Artemia* from the same hatch date and culture tank are very similar in size.)
3. Add 4L of fresh 100-g/L GSL media to 5 L plastic beakers.
4. Carefully transfer 20 *Artemia* to each beaker with a plastic transfer pipette, minimizing the amount of liquid transferred with each *Artemia*.
5. Aerate the beakers with an airstone (low air flow) to ensure even mixing and full air saturation and allow *Artemia* a minimum of 10 min to recover from handling.
6. Obtain a sample of *Dunaliella viridis* from the radioactive (Se-75) culture.
7. Centrifuge the sample of *Dunaliella viridis* at 8000 RPM in a microcentrifuge tube for 2 min.
8. Discard the radioactive supernatant.
9. Re-suspend the radioactive *Dunaliella viridis* in non-radioactive algae media.
10. Repeat steps 7-9.
11. Add an appropriate densitiy[1] of *Dunaliella viridis* grown in presence of Se-75 labeled selenium for 20 days.

[1] Algae cell density will be chosen to allow for sufficient Se-75 accumulation for accurate detection from preliminary experiments with the goal of feeding at densities as close to GSL algae densities as possible. Higher than desired algae cell density may have to be applied to allow for sufficient Se-75 uptake. So far our experiments have employed a cell density of approximately 2 mill cells/ml. Additional pilot experiments will determine the suited cell density for these experiments.

12. Take sub-sample of the *Dunaliella viridis* culture to measure selenium concentration in the *Dunaliella viridis* at the time of feeding.
13. Obtain sample of the *Dunaliella viridis* culture for accurate determination of cell density in the feeding experiment.
14. Obtain water samples from the feeding media at the beginning and end of the experiment to determine feeding rate.
15. After thorough mixing to avoid problems with settling perform manual cell count.
16. After 60 minutes[2], remove *Artemia* from feeding media and place them in individual gamma counting vials in 3 ml of GSL media.
17. Pass the vials through the gamma counter to determine Se-75 radioactivity in the newly fed *Artemia*.

[2] 30 and 60 min were used for the initial experiments - exact feeding time to be determined in additional pilot experiments.

Standard Procedures for Determination of Water-borne Selenium Assimilation Efficiency

1. Follow SOP for determination of water-borne selenium uptake steps 1-9. Use adult fully grown individuals.
2. Allow for a total of 48 hours of exposure (the longest exposure time we are comfortable with without feeding) to allow for selenium accumulation.
3. After 48 hours of exposure take a final water sample (100 µL) for determination of Se-75.
4. Carefully remove individual artemia with plastic transfer pipette and transfer them (individually) through a series of 3 rinses (10-15 mls each) of fresh media in a 6-well plate prior to placing them in individual gamma counting vials containing 3 mls of Se-75 free GSL media.
5. Pass these samples through a gamma counter for Se-75 determination in the live artemia.
6. After gamma counting, transfer individual artemia to separate 50-ml falcon tubes containing 30 ml GSL media. Feed animals daily and renew GSL media every other day.
7. At regular intervals (days apart), repeat steps 4-6 until significant depuration has been achieved.
8. Once depuration has been achieved and after a final rinse, carefully blot individuals dry on a paper towel and determine mass on weighing paper to nearest 10 µg.

9. Dilute the Se-75 stock as appropriate to measure cold Se on the GFAAS; then take three 10-μL samples of this diluted stock to be read on gamma counter.
10. Determine CPMs of all relevant samples on gamma counter: blank, initial water sample, blank, final water sample, 30 individuals, final water sample #2, 5 individuals, and diluted Se-75 stock.
11. Measure cold Se on GFAAS and determine specific activity of Se-75 stock by dividing: (cpm/L) / (μg Se/L) = cpm/μg Se.
12. Calculate Se accumulation and depuration according to: (cpm/individual) / (cpm/μg) = μg Se/individual.

Standard Procedures for Determination of Water-borne Selenium elimination

1. Follow SOP for determination of water-borne selenium uptake steps 1-9. Use adult fully grown individuals.
2. Allow for a total of 48 hours of exposure (the longest exposure time we are comfortable with without feeding) to allow for selenium accumulation.
3. After 48 hours of exposure take a final water sample (100 μL) for determination of Se-75.
4. Carefully remove individual artemia with plastic transfer pipette and transfer them (individually) through a series of 3 rinses (10-15 mls each) of fresh media in a 6-well plate prior to placing them in individual gamma counting vials containing 3 mls of Se-75 free GSL media.
5. Pass these samples through a gamma counter for Se-75 determination in the live artemia.
6. After gamma counting, transfer individual artemia to separate 50-ml falcon tubes containing 30 ml GSL media. Feed animals daily and renew GSL media every other day.
7. At regular intervals (days apart), repeat steps 4-6 until significant depuration has been achieved.
8. Once depuration has been achieved and after a final rinse, carefully blot individuals dry on a paper towel and determine mass on weighing paper to nearest 10 μg.
9. Dilute the Se-75 stock as appropriate to measure cold Se on the GFAAS; then take three 10-μL samples of this diluted stock to be read on gamma counter.

10. Determine CPMs of all relevant samples on gamma counter: blank, initial water sample, blank, final water sample, 30 individuals, final water sample #2, 5 individuals, and diluted Se-75 stock.
11. Measure cold Se on GFAAS and determine specific activity of Se-75 stock by dividing: (cpm/L) / (μg Se/L) = cpm/μg Se.
12. Calculate Se accumulation and depuration according to: (cpm/individual) / (cpm/μg) = μg Se/individual.

Standard Procedures for Determination of Dietary Selenium Assimilation Efficiency

1. Follow SOP for determination of dietary selenium intake steps 1-17.
2. After gamma counting, transfer *Artemia* to individual 15-ml falcon tubes containing 10 ml GLS media (100 g/l).
3. Feed the *Artemia* a Se-75-free diet and allow them to depurate fecal matter overnight.
4. Collect the *Artemia* from the 15-ml falcon tubes and recount individual *Artemia* for Se-75 as in steps 16 and 17.
5. Determine the wet weight of the individual *Artemia* and dispose.
6. Allow fecal matter in the 15-ml falcon tubes to settle; then siphon off 7 of the 10 mls of media.
7. Vortex the falcon tube now containing 3 ml of GSL media and fecal matter and rapidly transfer contents to a gamma counting vial.
8. Count these vials to determine the Se-75 content in the fecal matter.
9. Calculate dietary selenium intake from the specific Se-75 activity of the original algae culture medium and the initial Se-75 radioactivity in the *Artemia.*
10. Calculate the selenium assimilation efficiency from the Se-75 activity in the *Artemia* at the first and the second Se-75 determination. The difference equals the amount lost with fecal matter.
11. Calculate the dietary selenium uptake from the specific Se-75 activity of the original algae culture medium and the second Se-75 radioactivity measurement in the *Artemia*.

12. Validate the assimilation efficiency measurements by comparing the Se-75 lost between the initial and the final Se-75 activity measurements in the *Artemia* to the values detected in the fecal matter.

Waterborne Acclimation Experiment:

1. Remove ~100 adult, age-matched artemia from main culture tank and rinse in fresh media in a 200-ml beaker.
2. Add 1 L of 100-g/L GSL media to each of three 1-L tripour beakers.
3. Carefully transfer 30 artemia to each beaker with a plastic transfer pipette, minimizing the amount of liquid transferred with each artemia.
4. Gently aerate each beaker with capillary tubing to ensure even mixing and full air saturation.
5. Spike one beaker with an appropriate volume of Se-75 labeled Se stock (of known specific activity) to achieve 2 μg Se/L in the media. Spike another beaker with the same volume of unlabelled Se stock. The third beaker receives no addition of Se.
6. Take 3-mL initial water samples (in duplicate) from the Se-75 beaker and measure radioactivity on the gamma counter to verify exposure concentration.
7. Take duplicate 3-mL water samples daily from the Se-75 beaker, measure radioactivity, and spike with additional Se-75 labeled Se stock (or dilute with GSL media) to maintain 2 μg Se/L. Mirror the Se spikes and/or dilutions in the second beaker with unlabeled Se stock.
8. Feed each beaker daily with equal amounts of *Dunaliella viridis*. Feeding should be done 3-4 hours before water sampling and Se spiking to minimize uptake of Se by the algae cells.
9. Maintain waterborne exposures in the beakers for 2 weeks.

10. Perform waterborne uptake and depuration experiment with artemia exposed to 2 μg/L unlabeled Se (from second beaker) and artemia not exposed to Se in the media (from third beaker) according to **Standard Procedures for Se-75 Experiments – Uptake from the Water.** (Note: artemia from beaker containing Se-75 are not used in uptake and depuration experiment but simply serve to monitor exposure concentrations).

Dietary Acclimation Experiment:

11. Culture *Dunaliella viridis* in the presence of 1 μg Se/L (non-radioactive selenium) for 20 days. (This culture was prepared at the same time as the 1 μg/L Se-75 algae culture used in the dietary uptake experiment. Se spikes and dilutions made in the radioactive culture in order to maintain exposure concentrations were mirrored with unlabelled Se stock in the non-radioactive culture.)
12. Remove ~70 adult, age-matched artemia from main culture tank and rinse in fresh media in a 200-ml beaker.
13. Add 1 L of 100-g/L GSL media to each of two 1-L tripour beakers.
14. Carefully transfer 30 artemia to each beaker with a plastic transfer pipette, minimizing the amount of liquid transferred with each artemia.
15. Gently aerate each beaker with capillary tubing to ensure even mixing and full air saturation.
16. Feed each beaker equal amounts (normalized by absorbance at 750 nm to account for differences in culture density) of either non-radioactive Se-loaded *D. viridis* or normal *D. viridis* (not cultured in the presence of Se) daily for 2 weeks.
17. Remove 25 artemia from each beaker and transfer to beakers containing 4 L of 100-g/L GSL media.
18. Follow **Standard Procedures for Determination of Dietary Selenium Intake,** steps 5-17.